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Enzyme-coupled assays for simultaneous detection of nanomolar ATP, ADP, AMP, adenosine, inosine and pyrophosphate concentrations in extracellular fluids

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ABSTRACT

Purinergic signaling cascade includes the release of endogenous ATP and other agonists by chemical and mechanical stimuli, modulation of diverse cellular functions and subsequent ectoenzymatic inactivation. Basal release of extracellular purines and its physiological relevance remain controversial. Here we employed a combination of enzyme-coupled approaches for simultaneous bioluminescent (ATP, ADP, PP_i) and fluorometric (AMP, adenosine, inosine, hypoxanthine) measurements of ATP and its metabolites without additional manipulations or derivatization of sampled biological fluids. By using these sensing techniques, extracellular purines were determined in various cells and tissues both at resting and pro-inflammatory conditions. The results obtained revealed the ability of endothelial, lymphoid and tumor cells to maintain extracellular ATP, ADP and adenosine at certain characteristic nanomolar levels. By quantifying the amounts of endogenously released and/or exogenously applied purines and their metabolites, these sensing techniques may be applied for evaluating purine-converting pathways on the cell surfaces and also for *ex vivo* analysis of purine homeostasis in the intact tissues. Furthermore, we provide novel insight into the mechanisms underlying tumorigenic effects of ATP by demonstrating the ability of metastatic prostate carcinoma PC3 and breast cancer MDA-MB-231 cells to maintain PP_i, which derives from extracellular ATP in the course of nucleotide pyrophosphatase/phosphodiesterase reaction. Collectively, the results imply a complex pattern of nucleotide turnover where extracellular ATP, ADP and adenosine are maintained at steady-state levels *via* counterbalanced release and inactivation of ATP and other purines, and further suggest the importance of basal agonist release for continuous activation and/or desensitization of purinergic receptors.

1. Introduction

Extracellular purines are important signaling molecules regulating diverse responses in cardiovascular, nervous and other systems [1–3]. Purinergic signaling is thought to represent a multistep coordinated cascade, including stimulated release of endogenous ATP and ADP, triggering of signaling events *via* a series of ligand-gated P2X and G-protein coupled P2Y receptors and further nucleotide breakdown to adenosine. Cellular mechanisms underlying nucleotide release substantially vary depending on the cell type and stimuli applied and may particularly include cargo-vesicle trafficking and exocytotic granule secretion *via* vesicular nucleotide transporters; electrodiffusional movement through ATP-conducting anion channels or connexin/pannexins hemichannels; and facilitated diffusion by nucleotide-specific transporters of the ABC protein family [4–7]. Subsequent to the signal transduction, extracellular ATP and other nucleotides need to be rapidly inactivated to adenosine. General schemes of nucleotide hydrolysis have included a role for the enzymes of ecto-nucleoside triphosphate diphosphohydrolase (NTPDase) family, ecto-nucleotide pyrophosphatase/phosphodiesterase (NPP) family, ecto-5′-nucleotidase/CD73 and alkaline phosphatases [4,8,9]. The resulting adenosine in turn binds to its own nucleoside-selective G-protein coupled receptors [1,2] and finally, is inactivated on the cell surface [4] and/or uptaken by the cell *via* nucleoside transporters [10].

Recent advances in our understanding of the "release-signaling-inactivation" sequence provide significant justification for re-examination of the current models of purinergic machinery. Firstly, along with the common view of extracellular ATP as a ligand for P2 receptors, substrate for ecto-nucleotidases and source of adenosine, this nucleotide was shown to concurrently serve as a phosphoryl donor for counteracting ecto-adenylate kinase and nucleoside diphosphate kinase (NDPK)

Abbreviations: ADA, adenosine deaminase; APS, adenosine 5′-phosphosulfate; BSS, basal salt solution; HDMEC, human dermal microvascular endothelial cells; HRP, horseradish peroxidase; HUVEC, human umbilical vein endothelial cells; KRPG, Krebs Ringer phosphate glucose; NBT, S-(4-nitrobenzyl)-6-thioinosine; NDPK, nucleoside diphosphate kinase; NPP, nucleotide pyrophosphatase/phosphodiesterase; NTPDase, nucleoside triphosphate diphosphohydrolase; PNP, purine nucleoside phosphorylase; TLC, thin-layer chromatography; XO, xanthine oxidase

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reactions [4,11–13]. The co-existence of two counteracting, nucleotide inactivating and transphosphorylating, pathways represents a principally distinct route for appearance and tuned regulation of purinergic agonist levels on the cell surface. Secondly, along with transient release of nucleotides by various excitatory/secretory and non-excitatory tissues and cells under different mechanical, chemical and other stimuli, some cells constitutively release ATP at certain basal rates [5,14]. Continuous presence of extracellular ATP may have physiological implications for diverse cell responses, including regulation of blood flow, control of cell growth, neuronal activity, and response to pathogens [5]. Thirdly, innovative ATP-sensing assays provide independent lines of evidence that micromolar levels of ATP can be spatially confined in the pericellular space of lymphocytes [15], astrocytoma cells [16], platelets [17], epithelial cells [14,18], tumor cells [19] and other cell types [4,20], with only minor nucleotide portion being diffused and/or convected into the bulk medium. Fourthly, by using microelectrode biosensors, it was shown that other purines may appear in the extracellular milieu in response to hypoxia and ischemia *via* direct release of endogenous adenosine from hippocampal slices [21,22] as well as inosine and hypoxanthine from cerebellar cultures and slices [23]. Lastly, the importance of yet another product of extracellular ATP metabolism, pyrophosphate (PP_i), has been emerged recently, which particularly contributes to the regulation of bone and cartilage mineralization [24], calcification of rat vascular smooth muscle cells [25,26] and inhibition of caspase-1 activation and interleukin-1β release in the course of macrophage differentiation into anti-inflammatory phenotype [27].

Despite the significant progress in our understanding of extracellular signaling pathways, there is still a need for simple and reliable assays for detecting nanomolar levels of purinergic agonists and their metabolites in extracellular fluids. This study was undertaken (a) to elaborate a combination of enzyme-coupled bioluminescent and fluorometric assays appropriate for simultaneous measurement of the whole spectrum of adenine nucleotides, nucleosides and their metabolites in nanomolar range; and (b) by using these sensing techniques, to evaluate extracellular purine homeostasis in various cells and tissues during short-term (≤3 h) incubations under defined *in vitro* conditions. The results obtained revealed the ability of endothelial, lymphoid and tumor cells to maintain extracellular ATP, ADP and adenosine, as well as PP_i (in case of metastatic tumor cell lines) at certain characteristic nanomolar levels and further suggest the importance of these basal agonist levels for continuous activation and/or desensitization of purinergic receptors.

2. Materials and methods

2.1. Cell cultures

Human umbilical vein endothelial cells (HUVEC) were isolated from fresh umbilical cords of 8–10 different donors by treatment with collagenase, as described elsewhere [28]. HUVEC were cultured in endothelial cell growth medium EGM-2 BulletKit (Lonza, Walkersville, MD, USA) and were used in the experiments between passages 3 and 5. Human dermal microvascular endothelial cells (HDMEC) from three different donors were purchased from PromoCell GmbH (Heidelberg, Germany) and cultured in the accompanying endothelial cell growth medium MV with Supplement Mix. EAhy926 cell line (derived by the fusion of HUVEC with the continuous human lung carcinoma cell line A549), human leukemic T-cell lymphoblast line Jurkat (clone E6-1), prostate carcinoma cell line PC3, breast cancer cell line MDA-MB-231, and mouse B16-F10 melanoma cells were from ATCC (Manassas, USA). PC3 cells were maintained in Gibco Ham's F-12K medium containing 10% fetal calf serum. Other cells were cultured either in RPMI-1640 (Jurkat and EAhy926) or Dulbecco's Modified Eagle's (MDA-MB-231 and B16-F10) medium supplemented with 10% fetal calf serum, 4 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin. Endothelial and tumor cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and seeded onto 24-well tissue culture plate in complete media at a density ~50,000 cells/well for 24–48 h to reach the confluence.

2.2. Cell treatments

2.2.1. Study 1

For measurement of extracellular ATP, ADP and adenosine, monolayers of adherent cells or suspensions of Jurkat T-lymphocytes (2×10^5 cells/well) were washed and equilibrated at 37 °C in 24-well plate in the starting volume of 1 ml Krebs Ringer phosphate glucose (KRPG; comprising: 145 mM NaCl, 5.7 mM sodium phosphate, 4.86 mM KCl, 0.54 mM $CaCl_2$, 1.22 mM $MgSO_4$, and 5.5 mM glucose; pH 7.35). Aliquots of the bathing medium (260 μl) were carefully collected from the top of the wells at different intervals, either from non-stimulated cells or after cell stimulation with different drugs and heat-inactivated (5 min at 60 °C). The following drugs were dissolved from stock solutions and applied onto the cells at 1:100–1:500 dilutions: pro-inflammatory cytokine human recombinant tumor necrosis factor-α (TNFα, 200 U/ml) and interleukin 1β (500 U/ml) (R&D Systems), endotoxin lipopolysaccharide from *Escherichia coli* O111:B4 (LPS, 10 ng/ml), non-hydrolysable cAMP analogue dibutyryl cAMP (20 μg/ml), as well as inhibitor of nucleoside transport S-(4-nitrobenzyl)-6-thioinosine (NBT, 1 μM) (all from Sigma). Each drug was tested prior to the experimentation to ensure that it does not interfere with the employed luminometric and fluorometric assays. There was also no change in cell viability with any of these maneuvers.

2.2.2. Study 2

In the second study, cultured cells were incubated at 37 °C in 24-well plate in 1 ml of basal salt solution (BSS; comprising: 130 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgSO_4$, 25 mM HEPES, 5 mM glucose and 0.1% bovine serum albumin; pH 7.4) in the absence and presence of exogenous ATP or adenosine (2 μM). After 1-hour incubation, 700 μl of bathing medium was collected, heat-inactivated and assayed for ATP, ADP, AMP, adenosine, inosine, hypoxanthine and PP_i levels, as specified below.

2.3. Enzyme-coupled assay for fluorometric detection of AMP, adenosine, inosine and hypoxanthine

A novel sensing technique has been elaborated using specific mixture of enzymes sequentially converting purines into uric acid and H_2O_2, followed by fluorometric detection of the generated H_2O_2 (Fig. 1). Basic principle of the employment of enzyme-coupled reactions for determination of the purine bases had been proposed over 50 years ago [29] and since then the technique has been further developed and adapted for electrochemical detection of adenosine and inosine by using microelectrode biosensors [21,30,31]. Here, we employed a highly sensitive and stable probe 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red reagent) which reacts with H_2O_2 in the presence of horseradish peroxidase (HRP) with a 1:1 stoichiometry to produce fluorescent resorufin. Aliquots of conditioned medium (40–60 μl) were transferred into separate wells in white 96-well microplates containing various combinations of the following enzymes in a final volume of 170 μl appropriate buffer (either KRPG or BSS): 0.15 U/ml 5′-nucleotidase from *Crotalus adamanteus* venom, 0.3 U/ml adenosine deaminase (ADA, type IX from calf spleen), 0.25 U/ml bacterial purine nucleoside phosphorylase (PNP) and 0.15 U/ml microbial xanthine oxidase (XO; all from Sigma). After 20 min incubation at RT, 30 μl of H_2O_2-detecting mixture containing HRP (final concentration 1 U/ml, Sigma) and Amplex Red reagent (60 μM, Invitrogen, Molecular Probes) was added to the microwells, followed by measurement of the fluorescence intensity

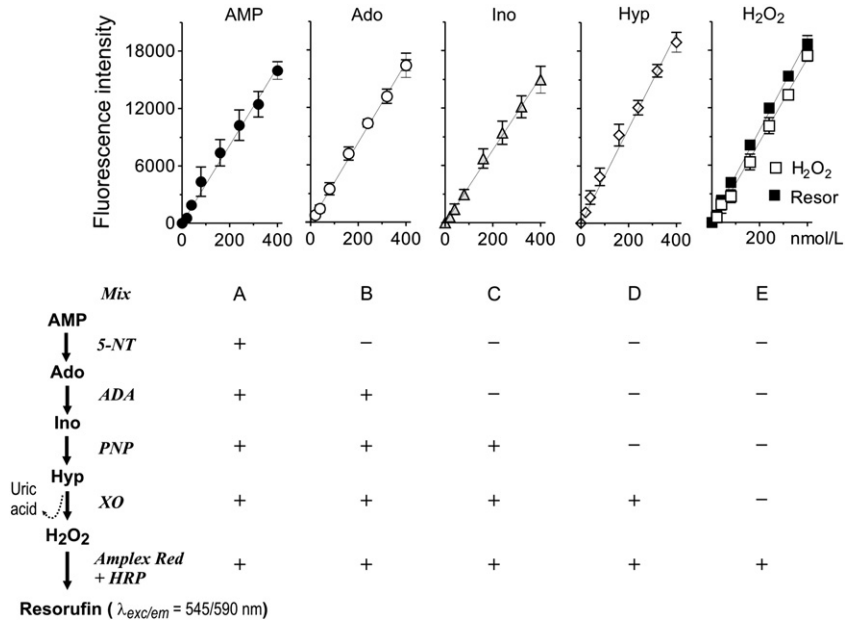

Fig. 1. Development of enzyme-coupled fluorometric purine-sensing assay. Addition of certain purine to the indicated mixtures of soluble enzymes caused its sequential conversion from AMP *via* adenosine (*Ado*), inosine (*Ino*) and hypoxanthine (*Hyp*) into uric acid and H_2O_2, with the latter metabolite being recorded fluorometrically using *HRP* and Amplex Red reagent. Differential recordings between wells "A and B", "B and C", "C and D", and "D and E" yield signals that are linearly related to the concentrations of AMP, adenosine, inosine and hypoxanthine, respectively. Calibration curves were generated for each experiment using identical coupled reactions with serial dilutions of AMP, adenosine, inosine, hypoxanthine and H_2O_2 standards, as well as the ultimate product of this catalytic chain, resorufin (*Resor*, Invitrogen, Molecular Probes).

at the emission and excitation wavelengths of 545 and 590 nm, respectively (Tecan Infinite M200, Salzburg, Austria). Particularly, the first well contained the complete enzymatic cascade ("mix A"), while in the parallel well the fluorescence was recorded using the same mixture but without 5′-nucleotidase (mix "B") (Fig. 1). A differential recording between wells "A" and "B" yields signals that are linearly related to the concentrations of AMP. For adenosine sensing, the background fluorescence determined in the absence of ADA (mix "C") has been subtracted from the fluorescence in well "B" containing the complete adenosine-converting cascade. Likewise, differential recordings between wells "C" and "D" and between "D" and "E" yield signals that are linearly related to the concentrations of inosine and hypoxanthine, respectively (Fig. 1).

2.4. Bioluminescent assays for quantification of ATP, ADP and PP_i in extracellular medium

For sensing ADP we took advantage of enzyme-coupled assay [32]. The principle of the assay is based on rapid ADP transphosphorylation with excessive amount of exogenous UTP in the presence of NDPK, with the generated product ATP being detected by a coupled luciferin/luciferase reaction (Fig. 2A). Specifically, 60-μl aliquots of conditioned medium were transferred into two wells of white non-phosphorescent 96-well microplate containing 90 μl distilled water without (A) or with (B) a mixture of UTP and NDPK from baker's yeast *Saccharomyces cerevisiae* (final concentrations 20 μM and 0.6 U/ml, respectively; Sigma) and incubated for 15 min. Subsequent to addition of 50 μl ATP-monitoring reagent (ATPLite assay kit, Perkin Elmer, Groningen, The Netherlands), luminescence of the samples was measured using a microplate reader (Tecan Infinite M200). Noteworthy, the measured luminescence in well "B" basically represents the integral value for ATP + ADP concentrations. Therefore, ATP was recorded in the parallel well "A" without UTP/NDPK reaction mixture and this value was subtracted from the above bioluminescence to yield specific ADP-dependent bioluminescence.

To quantify extracellular PP_i we adapted an enzyme-linked bioluminescent assay originally developed for monitoring inorganic pyrophosphate synthesis in chromatophores [33] and more recently modified

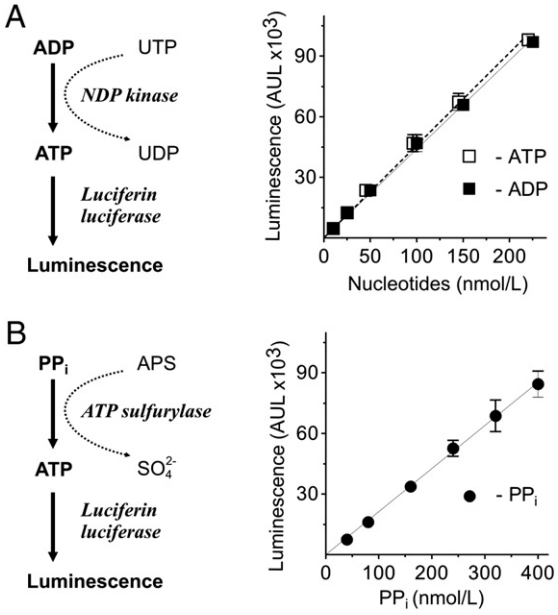

Fig. 2. Bioluminescent assays for quantification of ATP, ADP and PP_i. (A) Luciferin/luciferase assay was devised for simultaneous determination of ATP and ADP, with the latter nucleotide being detected by converting ADP into ATP in the presence of exogenous UTP and NDPK. (B) PP_i reacts with exogenous APS in the presence of ATP-sulfurylase to generate ATP, which can be measured then by a coupled luciferin/luciferase reaction. Calibration curves were generated for each experiment using identical coupled reactions with serial dilutions of ATP, ADP (A) and PP_i (B) standards, as illustrated on the right panels. Ordinates show arbitrary units of luminescence (AUL, $\times 10^3$).

for determination of nanomolar PP_i levels in extracellular fluids [25]. In this assay system, PP_i reacts with adenosine 5′-phosphosulfate (APS) in the presence of ATP sulfurylase to generate ATP which is measured by a coupled luciferin/luciferase reaction (Fig. 2B). Here, we modified and combined this bioluminescent approach with the above ATP/ADP-sensing technique, thus allowing us to detect nanomolar concentrations of ATP, ADP and PP_i within the same sample. Briefly, the aliquots of the medium (60 μl) were transferred into parallel Eppendorf tubes and mixed with 20 μl of BSS alone ("A") or supplemented with mixtures of UTP and NDPK (final concentrations 20 μM and 0.6 U/ml) ("B") or APS and ATP sulfurylase (20 μM and 0.01 U/ml; all from Sigma) ("C"). Samples were incubated either for 15 min at RT (tubes A and B) or 40 min at 37 °C (tube C) and then heat-inactivated. 70-μl aliquots of the mixture were transferred into white 96-well plate followed by addition of 10 μl 5× concentrated ATP-monitoring reagent and measurement of luminescence by Tecan Infinite M200. The differences in luminescence signals between wells "C" (ATP + PP_i) and "A" (only ATP) allowed quantifying the concentration of PP_i, which was converted into ATP through the ATP-sulfurylase reaction in the presence of APS. Likewise, the signal in well "A" was subtracted from the value in well "B" to yield specific ADP-specific bioluminescence. The linearity of the reaction as a function of ATP, ADP and PP_i concentrations is illustrated in Fig. 2.

2.5. Thin-layer chromatographic (TLC) analysis of purine-converting pathways

Cultured adherent cells or Jurkat T-lymphocyte suspensions (2×10^5 cells/well) were incubated at 37 °C in 24-well plate in the starting volume of 250 μl BSS containing 4 mM β-glycerophosphate and 10 μM ATP with tracer [2,8-^{3}H]ATP (Perkin Elmer) or 10 μM [2-^{3}H]adenosine (Amersham Biosciences) as initial substrates. Aliquots of the mixture (8 μl, ~5×10^4 dpm/spot) were periodically applied to an Alugram SIL G/UV254 TLC sheets (Macherey-Nagel, Duren, Germany). ^{3}H-labeled nucleotides and nucleosides were separated by using appropriate solvent systems and quantified by scintillation β-counting, as described earlier [34]. For autoradiographic analysis of [γ-^{32}P]ATP metabolism, PC3 and MDA-MB-231 cells were seeded overnight in 96-well flat bottom clear plates at a density 10,000 cells/well. Nucleotide metabolism was evaluated by incubating the adherent cells for 20 min at 37 °C in a final volume of 80 μl Dulbecco's Modified Eagle's medium containing 10 μM [γ-^{32}P]ATP (Perkin Elmer), 4 mM β-glycerophosphate and various unlabeled nucleotides. Aliquots of the mixture were spotted onto Polygram CEL-300 PEI sheets (Macherey-Nagel), separated by TLC with 0.75 M KH_2PO_4 (pH 3.5) as solvent and developed by autoradiography.

2.6. Ex vivo analysis of extracellular purines and PP_i in human umbilical cords

Fresh human umbilical cords (8-cm length) were sequentially washed with 10 ml of Hank's Buffered Salt Solution and twice with BSS. After infusion of 2 ml of BSS medium, the cords were clamped at both ends and incubated on a water bath for 20 min at 37 °C. The conditioned medium was collected by syringe, heat-inactivated (5 min at 60 °C) and assayed for purine nucleotides, nucleosides and PP_i as specified above. The same procedure was repeated by additional incubation of umbilical cords for 20 min with 2 ml BSS supplemented with 2 μM ATP or adenosine.

2.7. Statistical analysis

Each treatment was performed in duplicate or triplicate wells and data are expressed as mean ± SEM from at least three independent experiments.

3. Results

3.1. Cells maintain extracellular ATP, ADP and adenosine at certain characteristic nanomolar levels both at resting and pro-inflammatory conditions

Fig. 3 summarizes the measurements obtained with conditioned medium from various non-stimulated cells. ATP and ADP concentrations in the low nanomolar range (~2–5 nM) were detected in the wells with cultured HUVEC, HDMEC and EAhy926 cells. By contrast, tumor cells PC3, MDA-MB-231, B16-F10 and especially Jurkat T-lymphocytes maintained relatively high (up to 25 nM) nucleotide levels after 1-hour incubation, which slightly tended to decay after 3-hour equilibration. Concurrent measurement of adenosine also revealed extracellular concentrations of this nucleoside within 30–90 nM range (Fig. 3C). Surprisingly, except for ecto-5′-nucleotidase/CD73-negative Jurkat T-cells containing 16 ± 5 nM (n = 4) AMP, extracellular levels of this purine in other cells studied

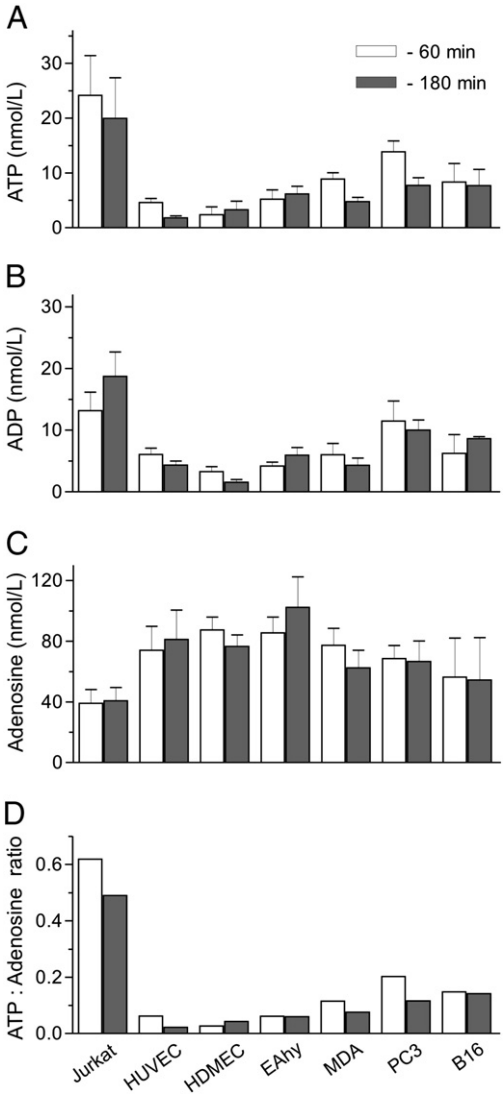

Fig. 3. Maintenance of basal extracellular purine concentrations by various cells. Cultures of Jurkat T-lymphocytes, HUVEC, HDMEC, EAhy926 (*Eahy*), MDA-MB-231 (*MDA*), PC3, and B16-F10 (*B16*) cells were incubated at 37 °C in 24-well-plate. Aliquots of the medium were collected after 60 (open bars) and 180 (closed bars) min and assayed for ATP (A), ADP (B) and adenosine (C) concentrations. The results are presented as mean ± SEM for at least 5 independent experiments performed in duplicate wells. Panel D represents the ratio of average extracellular ATP and adenosine concentrations shown in panels A and C, respectively.

were below the threshold detection limit of this assay (~5 nM or 1 pmol/well). Given that ATP and adenosine mediate diverse and often opposite responses [2,7], evaluation of the relationship between these key signaling molecules might provide additional information about the predominance of metabolic pathways for particular cell type. Interestingly, in comparison with Jurkat T-cells, HUVEC and HDMEC are characterized by relatively low ATP/adenosine ratio (Fig. 3D).

By having verified that cultured cells maintain extracellular purines at certain characteristic baseline levels, we then questioned whether various stimuli can trigger additional release of endogenous purines. Challenging of cultured HUVEC, EAhy926 and Jurkat T-cells for 1 h with pro-inflammatory agents TNFα, interleukin-1β and dibutyryl cAMP did not affect ATP and adenosine levels (Fig. 4), and similar results were obtained after 3-hour incubation (data not shown). In the presence of pro-inflammatory endotoxin LPS, extracellular concentrations of adenosine, but not ATP, were increased by ~50%. Treatment of HUVEC with inhibitor of nucleoside transport NBT was accompanied by 2-fold increase of adenosine (Fig. 4B). These findings are consistent with previous observations showing the abundant expression of equilibrative nucleoside transporters on vascular endothelium [10]. By contrast, the unchanged ATP and adenosine concentrations in Jurkat and EAhy926 cells after NBT treatment suggest the contribution of other cellular mechanisms to nucleoside homeostasis, most likely occurring *via* direct ectoenzymatic adenosine deamination [13]. Noteworthy, harsh pipetting of the medium directly down in three repetitions on the center of the wells with cultured cells was accompanied by significant (up to 4-fold) increases in extracellular ATP, but not adenosine, levels (data not shown).

3.2. Comparative analysis of purine-converting activities by using radio-TLC and novel enzyme-coupled sensing techniques

In the second study, cultured cells were incubated for 60 min at 37 °C with 2 μM ATP or adenosine followed by measurement of substrate clearance and metabolite formation. Noteworthy, by replacing KRPG medium with BSS in these particular assays we were able to determine, along with nucleotides and nucleosides, the levels of yet another important product of ATP metabolism, PP_i. Incubation of endothelial and tumor cells with ATP caused its partial breakdown and respective formation of dephosphorylated metabolites, ADP, AMP, adenosine, inosine and hypoxanthine, as well as PP_i (in case of tumor cells PC3 and MDA-MB-231) (Fig. 5A–D; right panels). Furthermore, concurrent TLC analysis of time-courses of [^{3}H]ATP metabolism (Fig. 5A–D; left panels) allowed us to compare this novel sensing approach with "classical" TLC-based enzyme assay. For instance, rapid clearance of exogenously applied ATP and its substantial conversion into adenosine, inosine and hypoxanthine by HUVEC correlates well with high ability of these cells to progressively hydrolyze [^{3}H]ATP into [^{3}H]nucleosides through sequential ecto-nucleotidase reactions (Fig. 5A). Incubation of Jurkat T-lymphocytes (Fig. 5B), PC3 (Fig. 5C) and MDA-MB-231 (Fig. 5D) with ATP triggered its predominant conversion into ADP without substantial increment in adenosine concentration, as revealed by using both radio-TLC and newly developed sensing techniques. The feasibility of this technique for assessment of purine-converting pathways was further ascertained by using ^{3}H-labeled or unlabeled adenosine as respective substrate. Fairly similar results were obtained by using radio-TLC and fluorometric enzyme-coupled assays, with both approaches showing the high ability of Jurkat T-cells and, to a lesser degree, other cells to convert adenosine *via* inosine to hypoxanthine *via* sequential ecto-ADA and PNP reactions (Fig. 6A–D).

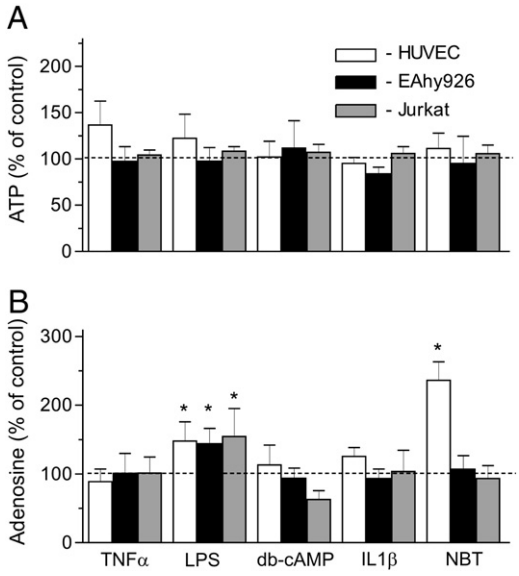

Fig. 4. Effect of pro-inflammatory stimuli on extracellular ATP and adenosine levels. Cultures of HUVEC, EAhy926 (*Eahy*) and Jurkat T-lymphocytes were challenged for 1 h with the following agents: TNFα (200 U/ml), LPS (10 ng/ml), dibutyryl cAMP (db-cAMP, 20 μg/ml), interleukin 1β (IL1β, 500 U/ml) and NBT (1 μM). Aliquots of the medium were collected and assayed for ATP (A) and adenosine (B). The results are presented as percentage of control purine concentrations measured in the absence of treatment. *$p<0.05$ as compared with control untreated wells.

3.3. Metastatic tumor, but not endothelial and lymphoid, cells display ecto-NPP activity and constitutively maintain PP_i in their vicinity

The revealed ability of PC3 and MDA-MB-231 cells to convert ATP into AMP and PP_i (see Fig. 5C,D) suggests the expression of NPP activities on these metastatic tumor cells. Additional analysis of basal PP_i levels further demonstrated that MDA-MB-231 and PC3 constitutively maintain extracellular PP_i at relatively high (>10 nM) and steady state levels, which substantially exceed those measured in HUVEC and Jurkat T-cells (Fig. 7A). For further substantiation of these results, we performed autoradiographic TLC imaging of nucleotide-converting pathways in these metastatic cells by using tracer [γ-^{32}P]ATP. As shown in Fig. 7B, incubation of PC3 cells with 10 μM [γ-^{32}P]ATP caused simultaneous generation of $^{32}PP_i$ and $^{32}P_i$ (lane 1) and these catalytic reactions were diminished in the presence of excessive amount (100 μM) of unlabeled ATP (lane 2). Joint addition of [γ-^{32}P]ATP and UDP induced their transphosphorylation into [γ-^{32}P]UTP and ADP through NDPK reaction (lane 3). The presence of backward ecto-adenylate kinase activity was ascertained by formation of [β-^{32}P]ADP during cell incubation with [γ-^{32}P]ATP and AMP (lane 4) and inhibition of this reaction by specific adenylate kinase inhibitor diadenosine pentaphosphate (Ap_5A; lane 5). Addition of [γ-^{32}P]ATP to another cell line, MDA-MB-231, also triggered its predominant breakdown into AMP and $^{32}PP_i$ (lane 6). Strikingly, contrary to the previously reported sole NTPDase1-mediated [γ-^{32}P]ATP hydrolysis into ADP and $^{32}P_i$ by vascular endothelial [28,32] and lymphoid [13,15] cells, the ability of PC3 and MDA-MB-231 cells to generate both $^{32}P_i$ and $^{32}PP_i$ indicates the co-expression of NTPDase and NPP activities on these metastatic cells. Collectively, these findings confirm that appearance of PP_i in the vicinity of tumor cells is coupled to ATP release and its subsequent hydrolysis *via* NPP reaction. Direct measurements also indicated that exogenous PP_i was very poorly cleared by these cells (data not shown), thus excluding the potential contribution of tissue non-specific alkaline phosphatases to extracellular PP_i homeostasis.

3.4. Evaluation of extracellular purine homeostasis in human umbilical cords

For testing whether the enzyme-coupled sensing techniques can be employed for measurement of purine levels in the tissues, we

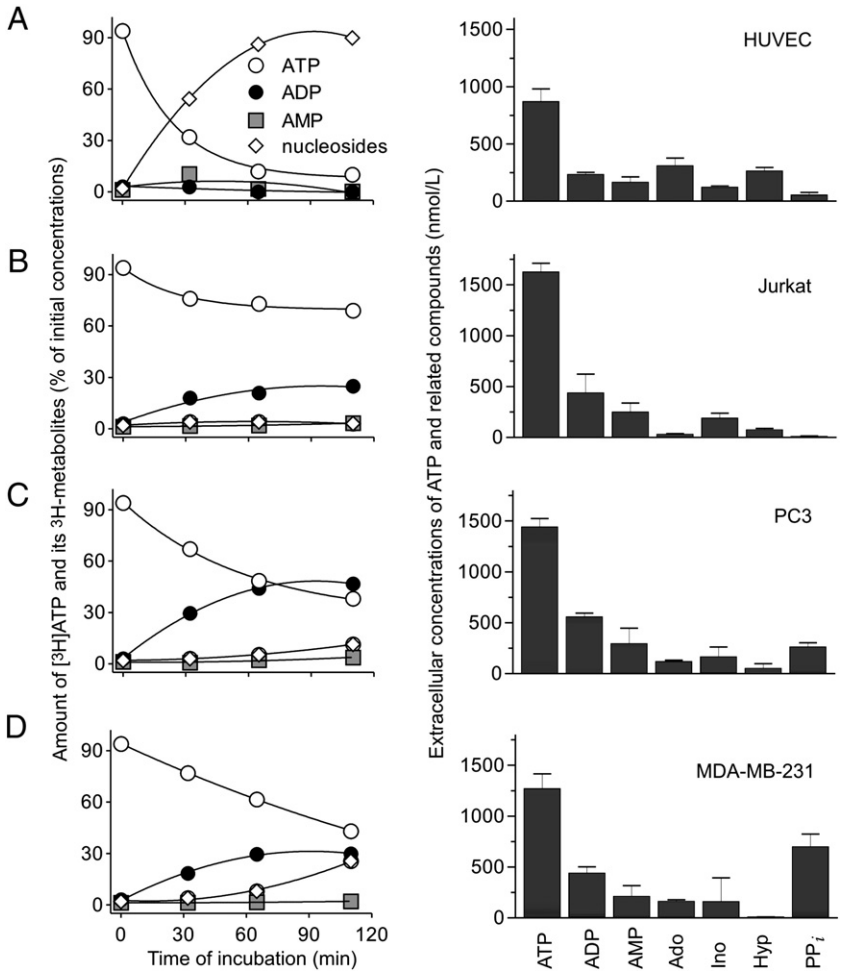

Fig. 5. Pattern of ATP metabolism by endothelial and tumor cells. Cultures of HUVEC (A), Jurkat T-lymphocytes (B), PC3 (C) and MDA-MB-231 (D) cells were incubated at 37 °C in 24-well-plate with exogenous ATP followed by measurement of the levels of ATP and related metabolites. Right panels show the concentrations of ATP, ADP, AMP, adenosine (*Ado*), inosine (*Ino*), hypoxanthine (*Hyp*) and PP_i in the conditioned medium collected after 60-minute incubation of the cells with 2 μM ATP and determined by using enzyme-coupled bioluminescent and fluorometric assays (mean ± SEM; n = 4–5). Cells were also incubated with 10 μM [^{3}H]ATP and aliquots of the medium were periodically collected and analyzed by TLC for the amounts of [^{3}H]ATP and its dephosphorylated metabolites. Left panels show the relative content of ^{3}H-labeled ATP, ADP, AMP and nucleosides (comprising the pooled adenosine, inosine and hypoxanthine fractions), expressed as percentage of initial concentrations. The graphs show mean data (n = 2); the standard error of the mean did not exceed the size of symbols.

incubated freshly isolated human umbilical cords with 2 ml BSS for 20 min. The conditioned medium was shown to contain very high concentrations of hypoxanthine (~3 μM) and nanomolar and submicromolar levels of adenosine and inosine. Certain amounts of PP_i (~10 nM) were also detected in the medium, whereas ATP and ADP were maintained at very low or undetectable levels (Fig. 8). Surprisingly, incubation of umbilical cords with 2 μM ATP or adenosine and subsequent analysis of the conditioned medium did not reveal significant shifts in the spectrum of measured metabolites (Fig. 8), thus suggesting the ability of umbilical endothelial cells to maintain nucleotide and nucleoside levels at certain levels *via* rapid turnover of the released purinergic agonists.

4. Discussion

Release of ATP and other agonists represents a critical component for initiating a purinergic signaling cascade. The novel findings of this paper are that 1) a combination of enzyme-coupled bioluminescent and fluorometric assays has been designed and adapted for concurrent measurement of the whole spectrum of adenine nucleotides, adenosine and related compounds (inosine, hypoxanthine and PP_i) within the same sample; and 2) basal extracellular levels of ATP and other physiologically relevant agonists ADP, adenosine and PP_i were shown to be maintained at certain characteristic nanomolar levels by cultured vascular endothelial, lymphoid and tumor cells and also in the intact tissues. Furthermore, by quantifying the amounts of exogenously applied purines and their metabolites, these sensing techniques may be employed for evaluating purine-converting ectoenzymatic pathways.

Luciferin/luciferase method has long been known as the most sensitive and selective approach for sensing ATP at various experimental settings, including off-line nucleotide detection in sampled biological fluids and real-time luminometry using soluble or cell-surface attached luciferase [4,5,16–18]. Moreover, the employment of particular mixtures of enzymes and substrates allowed to apply bioluminescent approach for simultaneous detection of ADP and ATP in pulmonary artery vasa vasorum endothelial cells [32], keratinocytes [35] and murine plasma [36,37], as well as extracellular ATP and PP_i in vascular smooth muscle cells [25,26]. Adenosine, inosine and/or hypoxanthine levels can also be detected by employing specific mixture of enzymes converting purines through the following sequence: adenosine → inosine → hypoxanthine → uric acid + H_2O_2, followed by electrochemical [21,23,30], fluorometric [38,39] or chemiluminescent [40] detection of the generated H_2O_2. Currently, the enzyme-coupled approaches are widely employed for real time measurements of ATP-

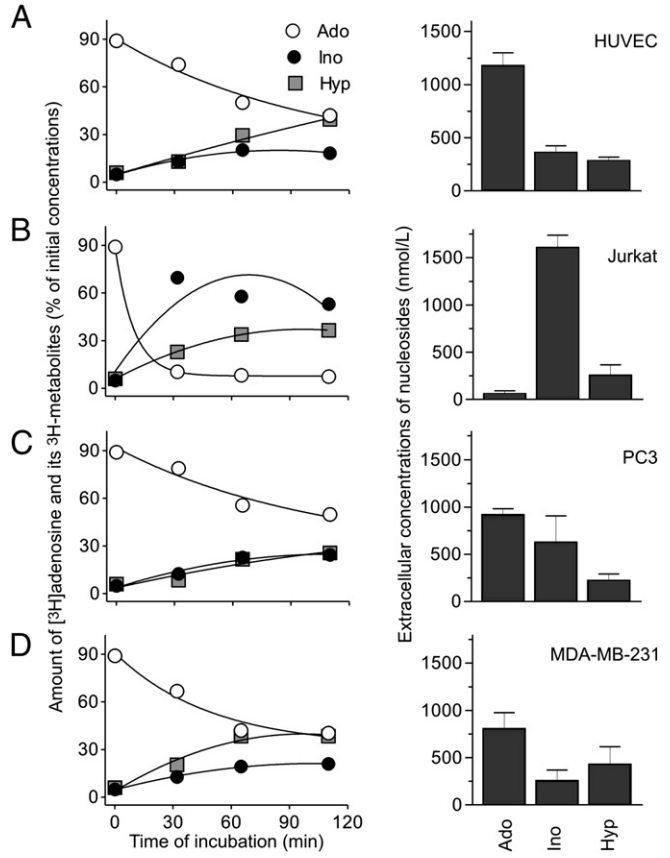

Fig. 6. Pattern of adenosine metabolism by endothelial and tumor cells. HUVEC (A), Jurkat T-lymphocytes (B), PC3 (C) and MDA-MB-231 (D) cells were incubated at 37 °C in 24-well-plate with adenosine. Right panels show the concentrations of adenosine (*Ado*), inosine (*Ino*) and hypoxanthine (*Hyp*) in the conditioned medium collected after 60-minute incubation of the cells with 2 μM adenosine and determined by using enzyme-coupled fluorometric assays (mean ± SEM; n = 4–5). Left panels show time-courses of [^{3}H]adenosine (10 μM) deamination by the cells and formation of ^{3}H-labeled inosine and hypoxanthine, as determined by TLC (n = 2).

and adenosine-releasing mechanisms by using microelectrode biosensors coated with appropriate ATP- or nucleoside-metabolizing enzymes, respectively [21,22,31,41]. The alternative sensing assays include pharmacological cell-based approaches for detecting ATP [42] and adenosine [43]; HPLC analysis of extracellular purines and bases [12,44,45] or their fluorescent 1,N^6-ethenoadenine derivatives [46,47]; continuous detection of ATP on living cells using atomic force microscopy with myosin-functionalized scanning tips [48] or tyrosine kinase-modified silicon nanowire chips [49]; fluorometric microscopic inspection of the cells loaded with ATP sensors luciferin or quinacrine [15,20], as well as PP_i detection by fluorescent or colorimetric chemosensors [50].

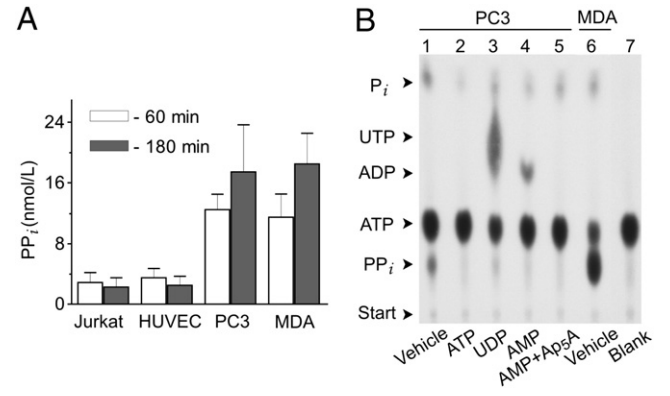

Fig. 7. Extracellular nucleotide metabolism and pyrophosphate levels in cultured cells. (A) The conditioned medium from cultured Jurkat, HUVEC, MDA-MB-231 (*MDA*) and PC3 cells was collected after 60 (open bars) and 180 (closed bars) min and assayed for PP_i using enzyme-coupled bioluminescent technique (mean ± SEM; n = 5–8). (B) PC3 and MDA-MB-231 (*MDA*) cells were incubated with 10 μM [γ-^{32}P]ATP in the absence (*vehicle*) or presence of 100 μM ATP, UDP, and AMP, as indicated. Some cells were pretreated for 20 min with 50 μM Ap_5A prior to addition of AMP and [γ-^{32}P]ATP. The *blank* shows the radiochemical purity of [γ-^{32}P]ATP in the absence of cells. Arrows indicate the positions of nucleotide standards, inorganic orthophosphate (P_i) and pyrophosphate (PP_i).

Despite the variety of sensing techniques, there is still a need for simple and reliable assay for concurrent analysis of purine nucleotides and nucleosides. By using a highly specific H_2O_2-sensing reagent Amplex Red, here we developed a novel assay for detecting nanomolar concentrations of AMP, adenosine, inosine and hypoxanthine. This fluorometric assay, when used in combination with other enzyme-coupled bioluminescent techniques, provides a unique possibility of quick and sensitive detection of ATP, ADP, AMP, adenosine and their metabolites in aqueous homogenous solution without additional manipulation, derivatization or dilution of sampled biological fluids. The results obtained are consistent with earlier observations showing the release of ATP by different secretory and non-excitatory cells at basal rates [4,5,11,14,47]. Furthermore, along with ATP, constitutive presence of ADP and adenosine in endothelial, lymphoid and tumor cells was demonstrated. Interestingly, among the studied cells, Jurkat T-cells displayed the highest ATP:adenosine ratio (see Fig. 3D) and these data corroborate previous observations that lymphoid and endothelial cells generally display counteracting ATP-regenerating/adenosine-deaminating and nucleotide-inactivating phenotypes, respectively [13]. Previous estimates of ADP [35,36,45,47] and adenosine [21,22,47] made in various cells, tissue slices and blood plasma also reported purine levels from low nanomolar to submicromolar range. It may be anticipated that maintenance of extracellular ADP and subsequent desensitization of $P2Y_1$ receptors predispose the described earlier impaired platelet aggregation and P-selectin expression in response to exogenous ADP [36,45], and also diminished antigen-presenting capacity and ADP-dependent death of epidermal dendritic cells [35]. Likewise, persistent activation of the A_2 receptors by endogenous adenosine may lead to their partial desensitization in various cell types and impaired cAMP responses to exogenously applied adenosine [32,47].

Unlike well-characterized ATP release under various stimuli, cellular mechanisms responsible for maintenance of ATP and other purines at basal levels remain largely unknown. One interesting hypothesis is that basal nanomolar extracellular ATP level basically reflects a minor fraction (so-called "purinergic tone" [11]) of the actual mass of micromolar pericellular nucleotide pool, which is localized in the immediate cell vicinity and exchanged with bulk phase *via* dissociation–association mechanisms [4,15–17]. Given that apparent K_m values for purinergic ectoenzymes lie within micromolar range [9,28], we also do not exclude that low nanomolar concentrations of extracellular purines are below the threshold level required for their efficient binding to the substrate-binding active centers of the enzymes. It may particularly explain why baseline levels of ATP and other purines remained essentially constant throughout the 3-hour incubation period, even despite the presence of high ecto-nucleotidase and ADA activities on the cells studied (see Figs. 6 and 7; also [13]). At the same time, induction of additional release of ATP by different mechanical or chemical stimuli would strongly accelerate the catalytic rates of purinergic enzymes and rapidly restore extracellular ATP and its metabolites to basal steady-state levels, thereby preventing excessive activation and/or desensitization of purinergic receptors. Indeed, except for significant LPS-mediated increase in adenosine, application of other pro-inflammatory agents did not affect extracellular levels of ATP and adenosine (see Fig. 4).

Besides *in vitro* analysis of cell cultures, these sensing techniques can also be applied for detecting purine levels in the tissues, as ascertained by *ex vivo* experiments with human umbilical cords

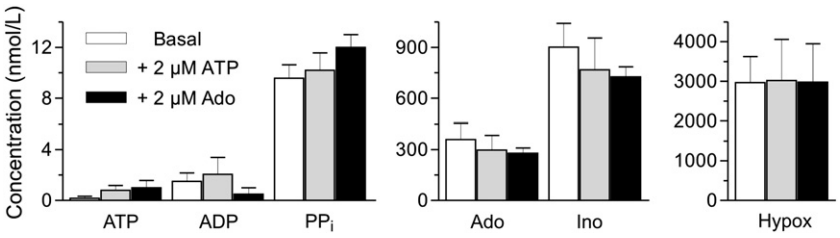

Fig. 8. *Ex vivo* analysis of purine nucleotides, nucleosides and PP_i in human umbilical cords. The concentrations of ATP, ADP, PP_i, adenosine, inosine and hypoxanthine were determined in the conditioned "intracordial" fluid obtained after 20-minute incubation of fresh umbilical cords with BSS medium, either alone (*basal*) or supplemented with 2 μM ATP or adenosine (mean ± SEM; n = 3–5).

(see Fig. 8). Assuming that endothelial lining primarily contributes to the measured "intracordial" homeostasis, the obtained data on undetectable ATP/ADP and high nanomolar adenosine/inosine levels correlate well with data on predominant nucleotide inactivating/adenosine generating phenotype in cultured HUVEC and other vascular endothelial cells (see Fig. 5A; also [12,13]), and are basically consistent with current view of important role of adenosine in the maintenance of endothelial barrier function [10,32,46]. As for the revealed ability of umbilical cords to maintain nanomolar PP_i and micromolar hypoxanthine levels (see Fig. 8), it presumably reflects the existence of pyrophosphate [24] and hypoxanthine [34,44] "pools" freely circulating in human blood and other extracellular fluids.

Another important application of these sensing assays may include the assessment of ectoenzymatic purine-converting pathways (see Figs. 6 and 7), thus providing potential alternative to TLC assays, which are widely employed for measurement of purinergic activities in various cells and tissues [13,37,51]. Strikingly, contrary to TLC analysis of the conversion of radiolabelled substrates, current measurements quantify a bulk pool of both exogenously applied and endogenously released purines and their metabolites. Moreover, by using these sensing assays we revealed the ability of metastatic prostate carcinoma cells PC3 and breast cancer cells MDA-MB-231 to maintain nanomolar levels of PP_i, which primarily derives from endogenous ATP in the course of NPP reaction. Current models consider extracellular ATP as a potent tumorigenic agent triggering diverse tumor-promoting or -suppressive (depending on the tumor type) responses *via* direct activation of P2 receptors and/or subsequent nucleotide breakdown into adenosine [2,51,52]. So far, extracellular ATP-PP_i axis has been rather neglected as a potential mechanism regulating migration and invasion of metastatic cancer cells. Interestingly, a broadly employed class of synthetic analogues of PP_i, bisphosphonates, has been widely used for preventing bone resorption and osteoporosis, as well as for treatment of malignancies associated with bone metastasis, including breast and prostate cancers [24,53,54]. Most of these therapeutic effects are thought to be mediated through the inhibition of mevalonate/cholesterol biosynthesis or interference with other ATP-dependent intracellular pathways. Given that some bisphosphonates (specifically, non-nitrogen containing clodronate and etidronate) can substitute for PP_i by metabolic incorporation into non-hydrolyzable ATP analogues [54], it would be attractive to hypothesize that anti-tumor effects of these drugs are defined, at least in part, by interference with the function of the enzymes involved in extracellular PP_i homeostasis.

In summary, the results obtained imply a complex pattern of nucleotide turnover where different endothelial, lymphoid and tumor cells constitutively maintain ATP, ADP and adenosine at characteristic steady-state levels *via* counterbalanced release and inactivation of endogenous ATP and other purines. Furthermore, here we provide a novel insight into the mechanisms underlying tumorigenic effects of ATP by demonstrating the ability of metastatic breast and prostate cancer cell lines to maintain PP_i, which primarily derives from extracellular ATP in the course of NPP reaction. Simultaneous measurement of ATP, ADP, AMP, adenosine, inosine and PP_i within low nanomolar ranges may provide an extremely useful tool for further understanding the signal transduction pathways coordinately triggered by these agonists at different (patho)physiological states.

Acknowledgements

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